

General Structure of Heterobifunctional Linkers



R = Alkyl, cycloalkyl, cycloalkyl-alkyl, aromatic, alkyl-aromatic, stilbene, heterocyclic, alkyl-heterocyclic, CH₂CH₂-O-, alkyl-CH₂CH₂-O-alkyl, CH₂-CH=CH-, CH₂-NHCO, alkyl-NHCO-alkyl, CH₂CH₂-S-, CH₂CH₂-NH-, Long Chain Alkyl Amino, etc.

X = NH₂, succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol,

Y = Biotin

= Biotin/Avidin

= Biotin/Streptavidin (SA)

= Alkaline Phosphatase (AP)

= Casein

= beta-Lactamase

= BSA

= IgG

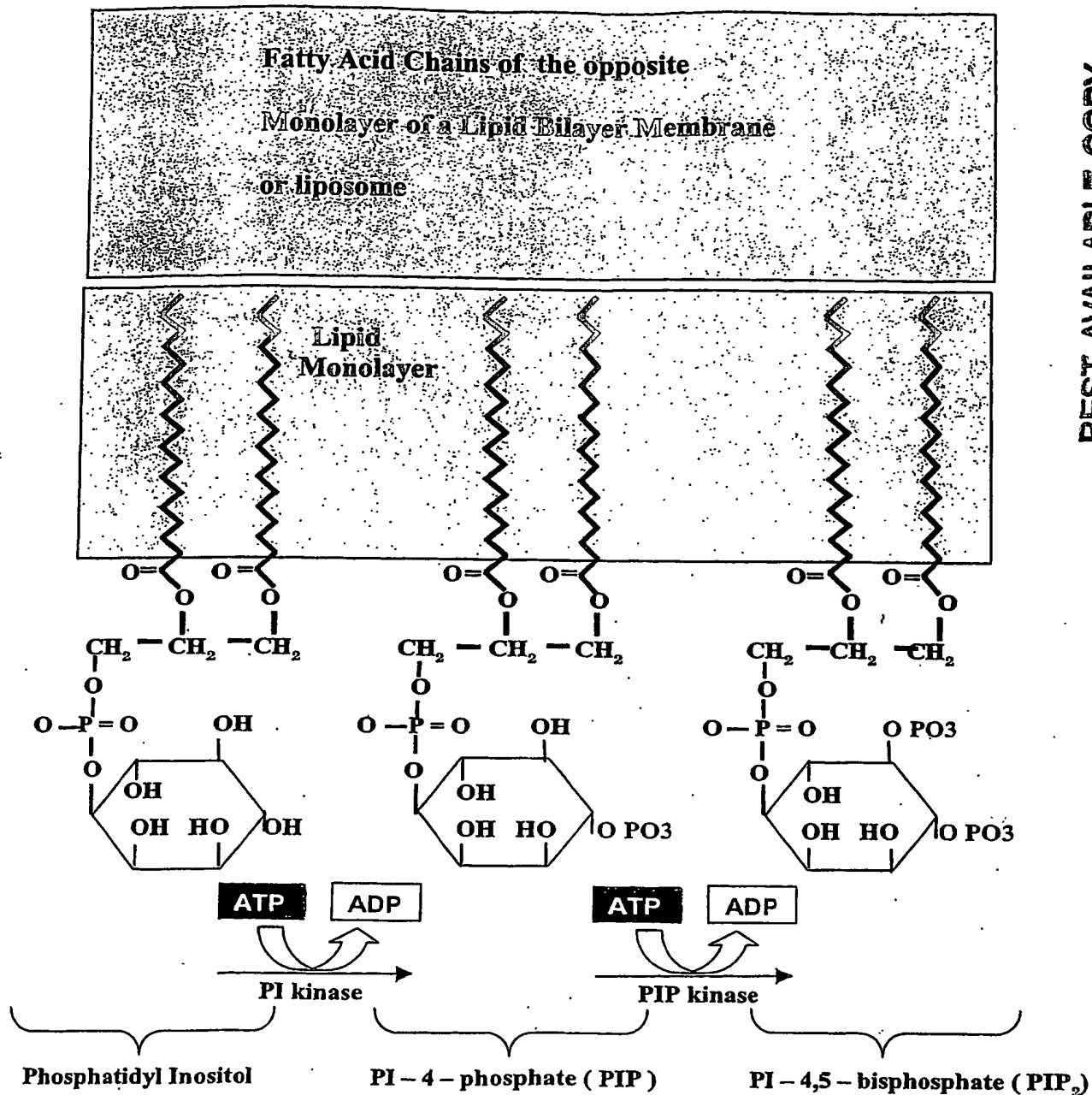
= Avidin-AP

= Streptavidin-AP

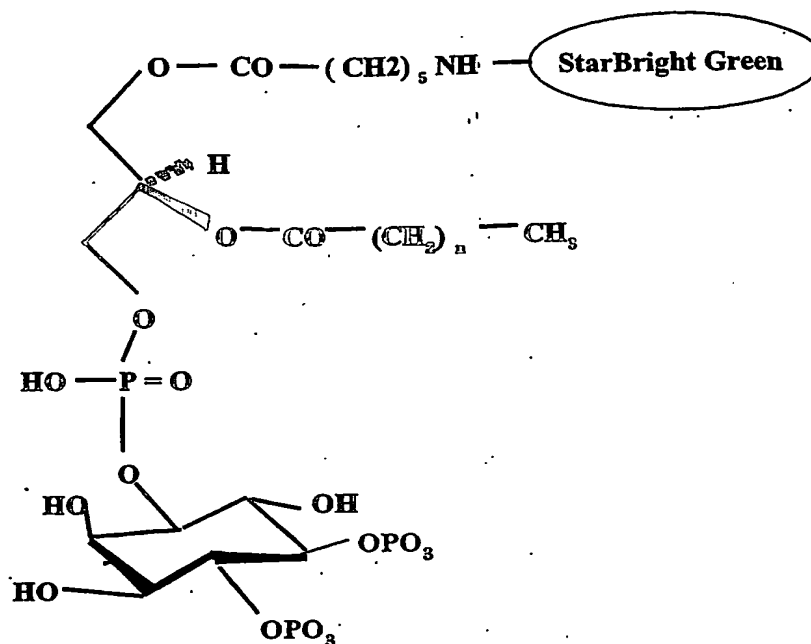
= Biotin or Streptavidin complexed with :

Glycoproteins, enzymes, antibodies, DNA, RNA, peptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and other solid surfaces such as microtitre plates, glass (silicon) plates, and any other polymer comprised of active functions, for example, -OH, -NH₂, -SH, succinimide, maleimide groups.

Figure 1. General chemical structure and compositions of the heterobifunctional linkers of the Present Invention

Figure 2. Classification of Kinases and Phosphatases by Target Structure**Figure 2a . a) representative water insoluble target and sites of specific actions of lipid kinases. Phosphatidyl Inositol and the Site specific actions of two lipid kinases**

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STARBRIGHT GREEN - PHOSPHATIDYLINOSITOL- 4,5- BISPHOSPHATE
[STARBRIGHT GREEN - PtdIns(4,5)P2]

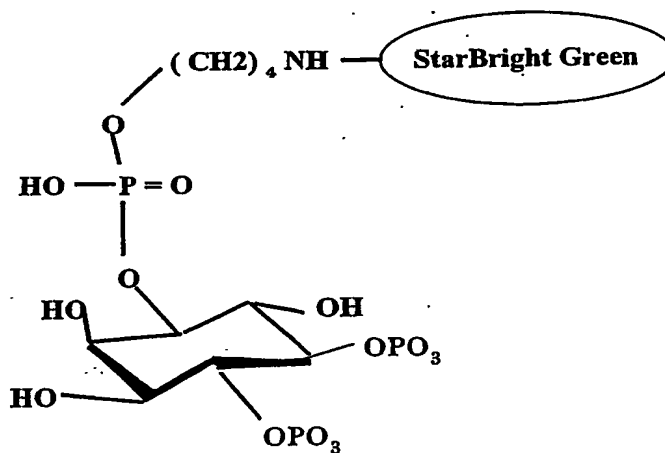


Figure 2.b. Water soluble lipid kinase target substrates: above, an example of the water soluble, StarBright-labeled derivatives of phosphatidyl inositol and its phosphorylated products. Alternative target substrates may be the single fatty acyl chain 1-StarBright Green -*myo*-inositol -1 phosphate lithium salts shown below and described in the text.

Arg - Phe - Ala - Arg - Lys - Gly - Ser - Leu - Arg - Gln - Lys - Asn - Val - COOH

OH

Arg - Phe - Ala - Arg - Lys - Gly - Ser - Leu - Arg - Gln - Lys - Asn - Val - COOH

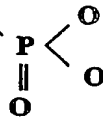


Figure 2. c. Peptide Target Substrate Phosphorylation -

The pseudosubstrate of Protein Kinase C-alpha and the site specific Phosphorylation of Serine by the PKC isozyme, PKC-theta

HO - CCA - ATC - TCA - TCT - TGT - TTT - CTG - CG - SPACER - StarBright Green

ATP, T4 nucleotide kinase, Ph 7.4

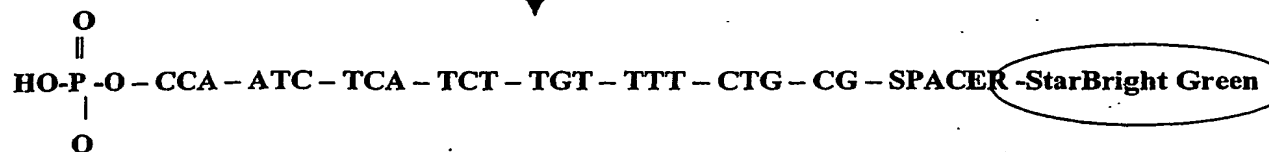
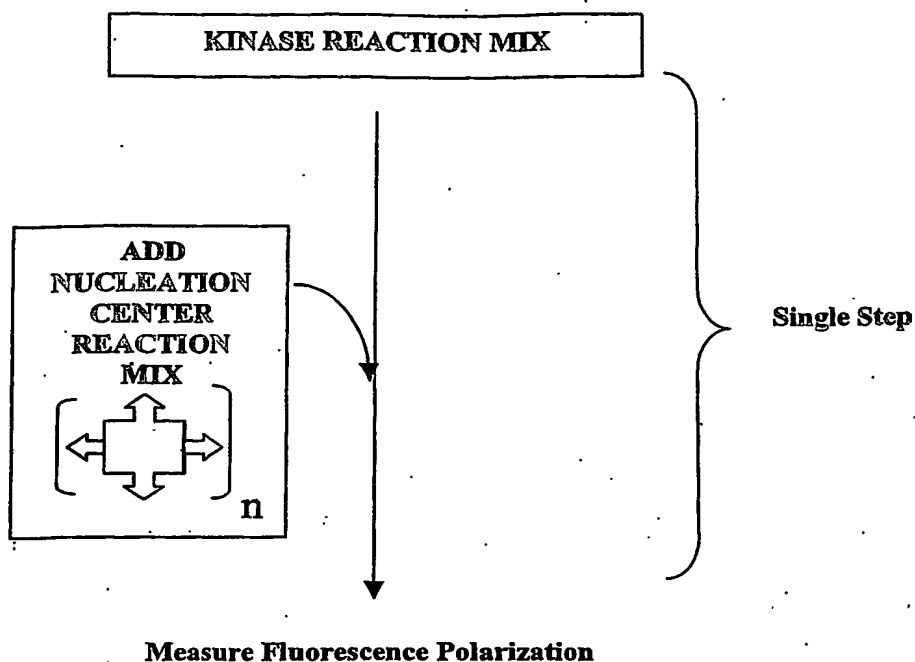


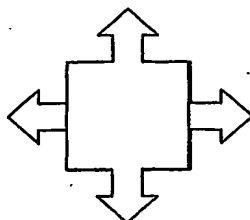
Figure 2.d. Oligonucleotide Target Substrate Phosphorylation -

The beta-actin target of T4 nucleotide kinase and the terminal phosphorylation of the oligonucleotide by the kinase

a) Single Step *Homogeneous* Assay using the rapid reaction method of the Present Invention

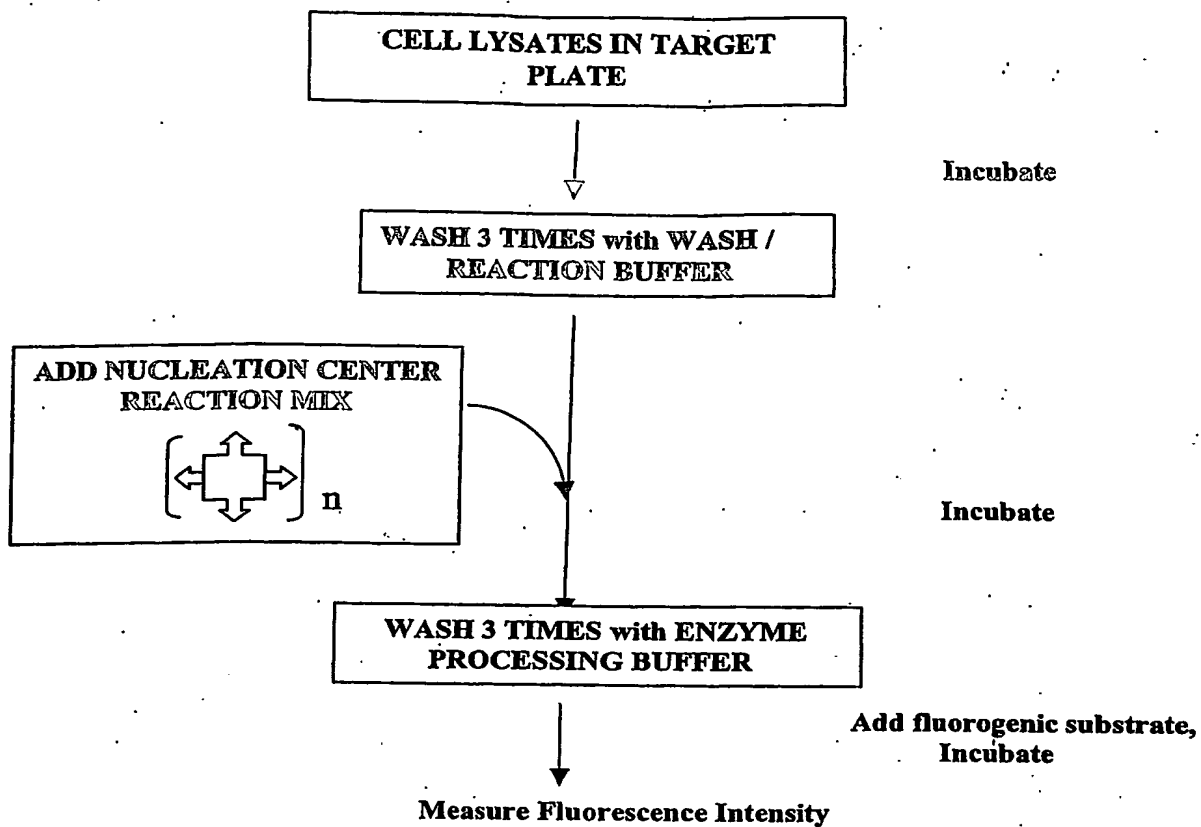


b) the "Nucleation Effect" in which multiple heterobifunctional linkers are attached to High Molecular weight core molecules such as avidin or another polymer to create a multi-valent reaction center that serves to enhance reaction rates,



where the square at the center represents the high molecular weight core that is conjugated to multiple copies ($n > 2$) of the heterobifunctional linkers (arrow heads) shown in Figure 1.

Figure 3. Schematic diagram (a) of the single step *homogeneous* assay method based upon the "nucleation effect" of the present invention and an idealized diagram (b) illustrating the nucleation effect itself;

a) Multi- Step *Heterogeneous* Assay of the Present Invention

b)

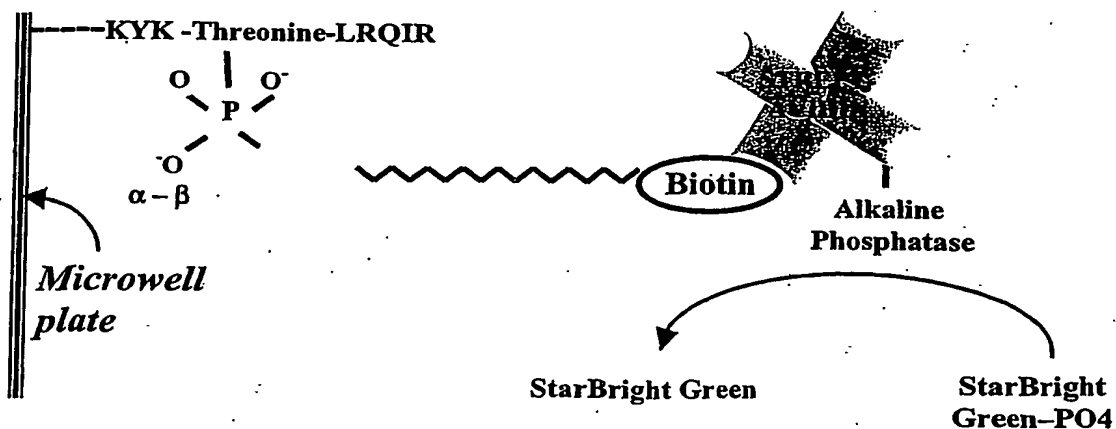


Figure 4. Schematic diagram (a) and mechanism (b) of the *heterogeneous* assay method : based upon the nucleation effect of the present invention

Phosphoramidate Chemistry For Developing Fluorescence Polarization Based Protein Kinase Assays

Schematic Representation of Steps Involved:

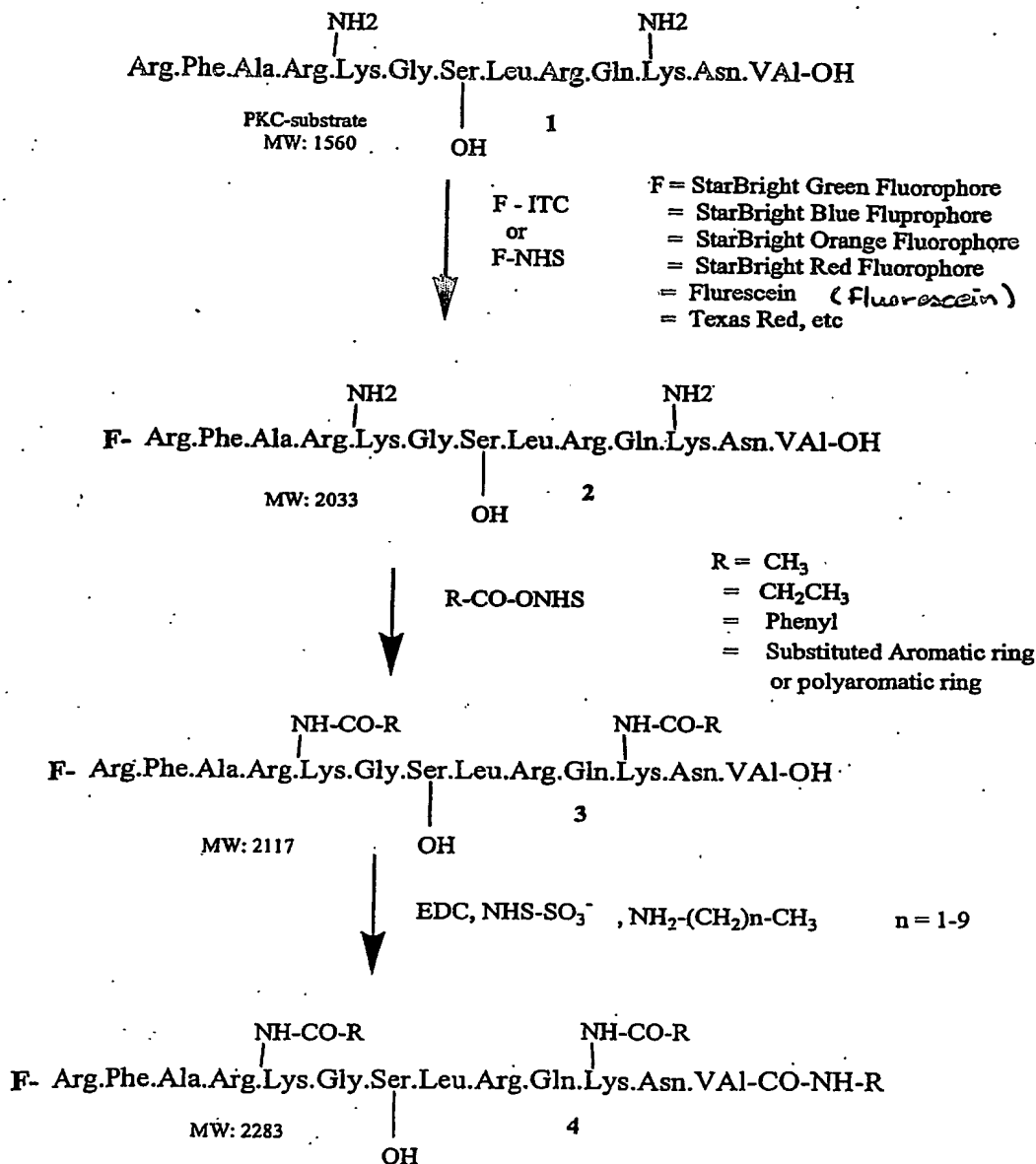


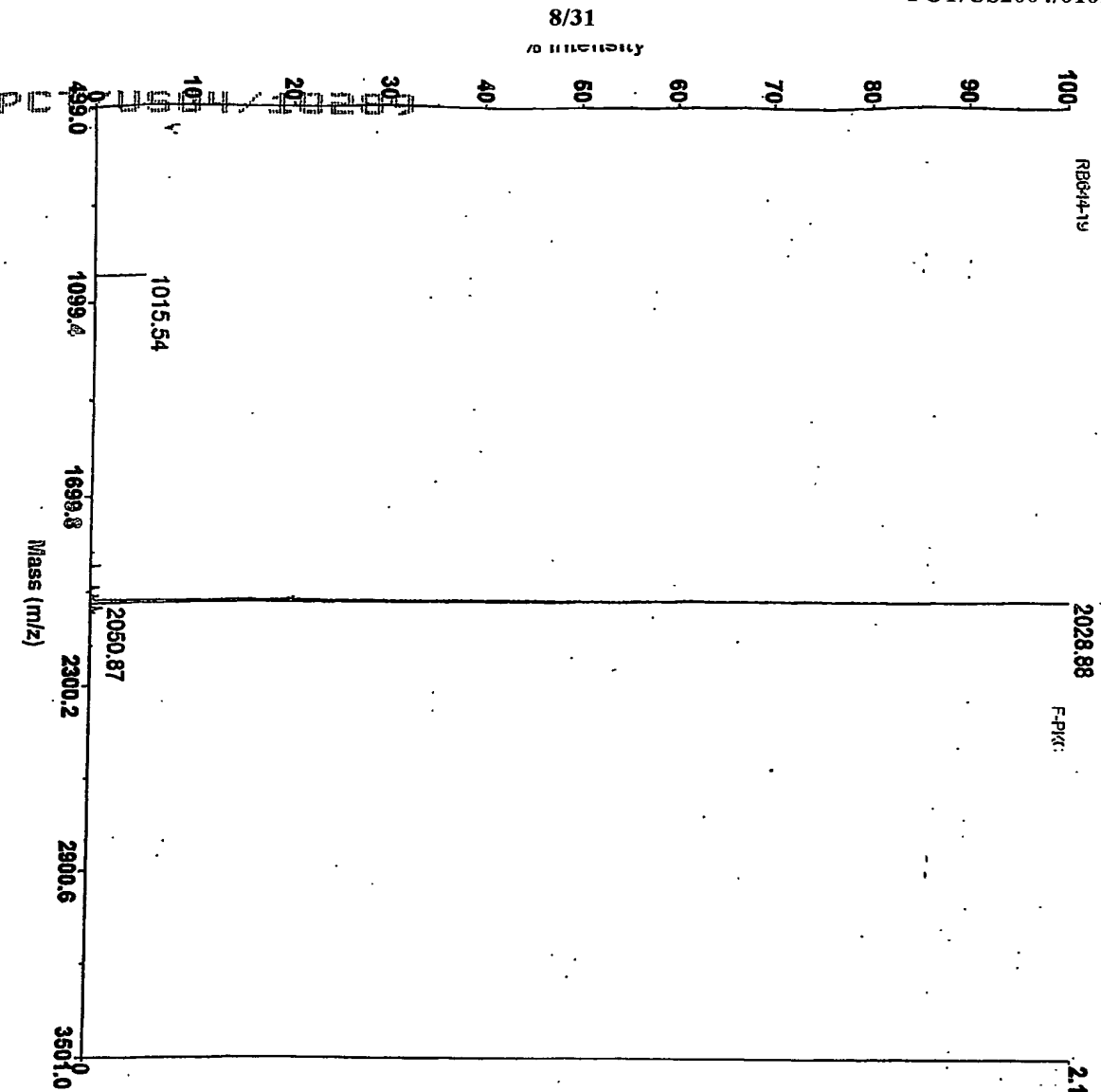
Figure 5. Novel protocols for blocking potentially reactive -NH₂ and -COOH groups on peptide targets of the present invention

Applied Biosystems Voyager System 1197

Voyager Spec #1[BP = 2028.9, 20777]

PCT/US2004/010289

WO 2004/089295



Mode of operation:
Extraction mode:
Polarity:
Acquisition control:

Linear
Delayed
Positive
Manual

2.1E+4 Accelerating voltage:

20000 V

Grid voltage:

95%

Guide wire O:

0.05%

Extraction delay time:

200 nsec

Acquisition mass range:

500 - 3500 Da

Number of laser shots:

100/spectrum

Laser intensity:

1548

Laser Rep Rate:

20.0 Hz

Calibration type:

Default

Calibration matrix:

8-Cyano-4-hydroxynaphthoic acid

Low mass gate:

500 Da

Digitizer start time:

14.258

Bin size:

2 nsec

Number of data points:

11636

Vertical scale:

1000 mV

Vertical offset:

0%

Input bandwidth:

150 MHz

Sample well:

01

Plate ID:

1

Serial number:

1197

Instrument name:

Voyager-DE

Plate type filename:

C:\VOYAGER\100 well plate.pil

Lab name:

PE Biosystems

Absolute x-position:

1398.02

Relative x-position:

47085.3

Relative y-position:

-189.475

Shots in spectrum:

212.193

Source pressure:

100

Mirror pressure:

1.178e-006

TC2 pressure:

0

TIS gate width:

0.0134

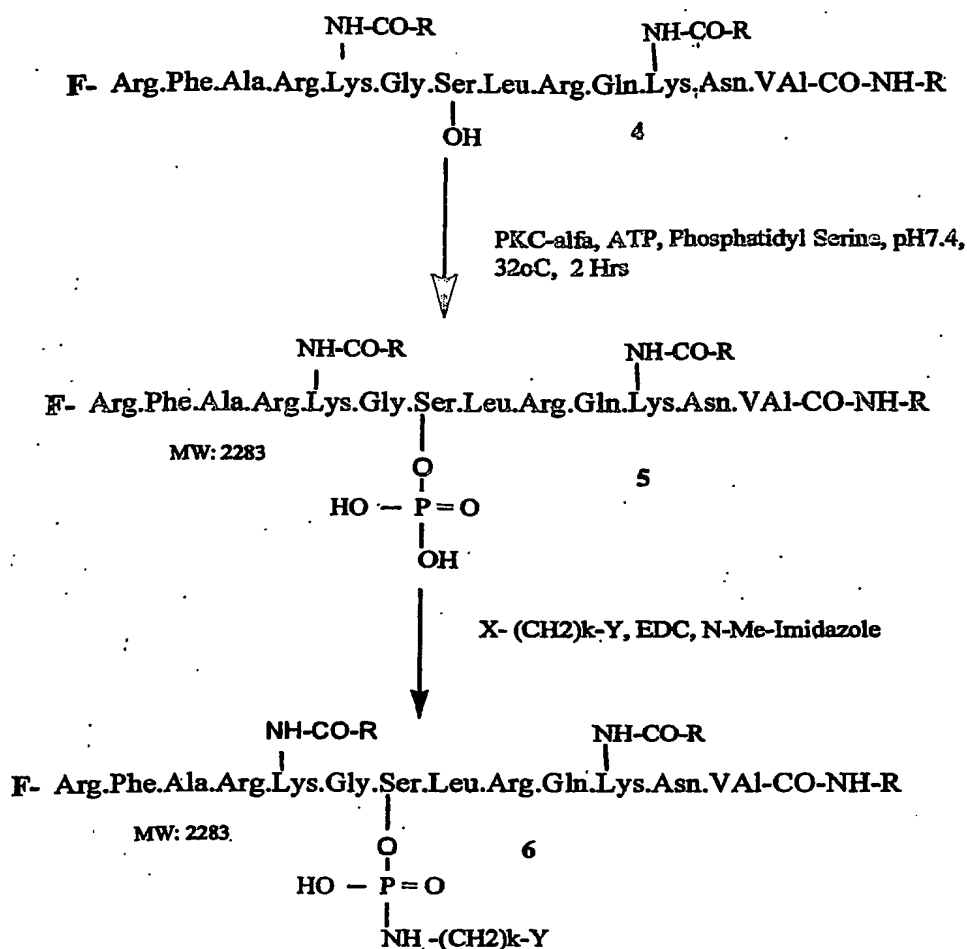
TIS flight length:

30

Figure 6.

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c:\agent\RAM\RB844-19\Frac8-7 August23, 02_0001.dcl

Maldi-Mass spectrum of the PKC-peptide target labeled with fluorescein at its N-terminal for the kinase activities of the isoforms of Protein Kinase C (PKC)



Where k = 1-15

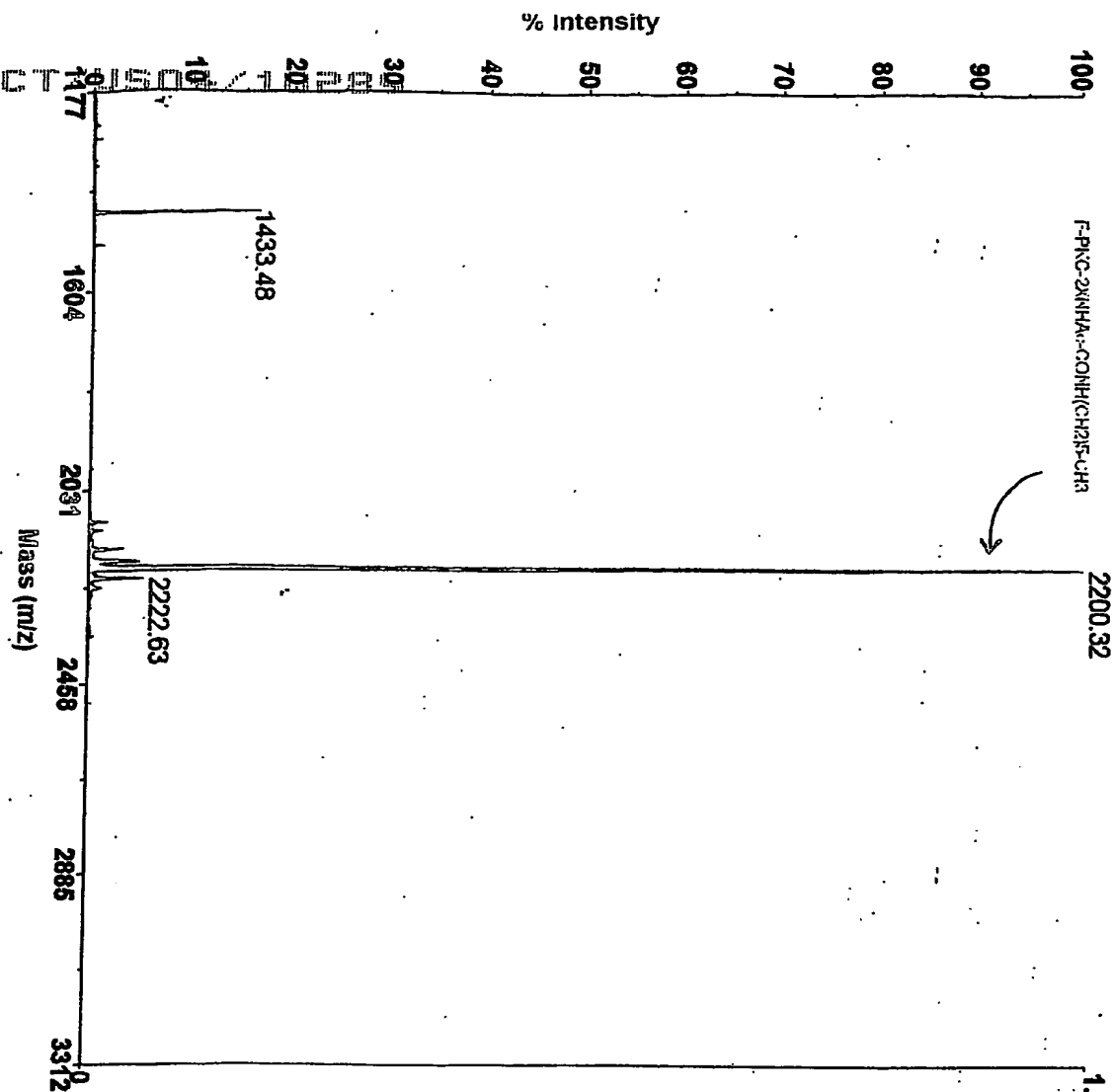
and Y = biotin, biotin-avidin complex, biotin-streptavidin complex, avidin-alkaline phosphatase (AP) conjugate, or unconjugated AP, b-Lactamase, Casein, or any other large molecular weight, including but not limited to antibodies, and derivatized particles.

Figure 7. Protocol and chemistry of the present invention for the formation of phosphor-amidates used in the detection of phosphoryl groups using the Nucleation Centers and rapid assay methods and phosphoramidate Chemistry I of the present invention

10/31

Applied Biosystems Voyager System 1197

Voyager Spec #11BP = 2199.9, 101171



1.0E+4

Mode of operation:
Extraction mode:
Polarity:
Acquisition control

Linear
Delayed
Positive
Manual

Accelerating voltage:
Grid voltage:
Guide wire O:
Extraction delay time:

20000 V
95%
0.05%
200 nsec

Acquisition mass range:
Number of laser shots:
Laser intensity:
Laser Rep Rate:

500 - 5000 Da
100/spectrum
1605
20.0 Hz

Calibration type:
Calibration matrix:
Low mass gate:

Default
a-Cyano-4-hydroxycinnamic acid
500 Da

Digitizer start time:
Bin size:
Number of data points:

14.258
2 nsec
16284

Vertical scale:
Vertical offset:
Input bandwidth:

1000 mV
0%
150 MHz

Sample well:

56

Plate ID:

JEFF

Serial number:

1197

Instrument name:

Voyager-DE

Plate type filename:

C:\VOYAGER\100 well plate.pil

Lab name:

PE Biosystems

Absolute x-position:

26803.1

Absolute y-position:

21753.4

Relative x-position:

-184.412

Relative y-position:

-154.076

Shifts in spectrum:

100

Source pressure:

1.332e-006

Mirror pressure:

0

TC2 pressure:

0.01227

TIS gate width:

30

TIS flight length:

940

quibed 11/23/00, April 11, 2002

voyager\Rebecca 621RH621-28 HPLC F1_0091.dat

Figure 8a: MALDI-MS of fully protected fluoresceinated PKC peptide target that potential reactive sites blocked as described in figure 5

27, March 10, 2003

11/31

Applied Biosystems Voyager System 1197

Voyager Spec #11BP = 2279.6, 3814]

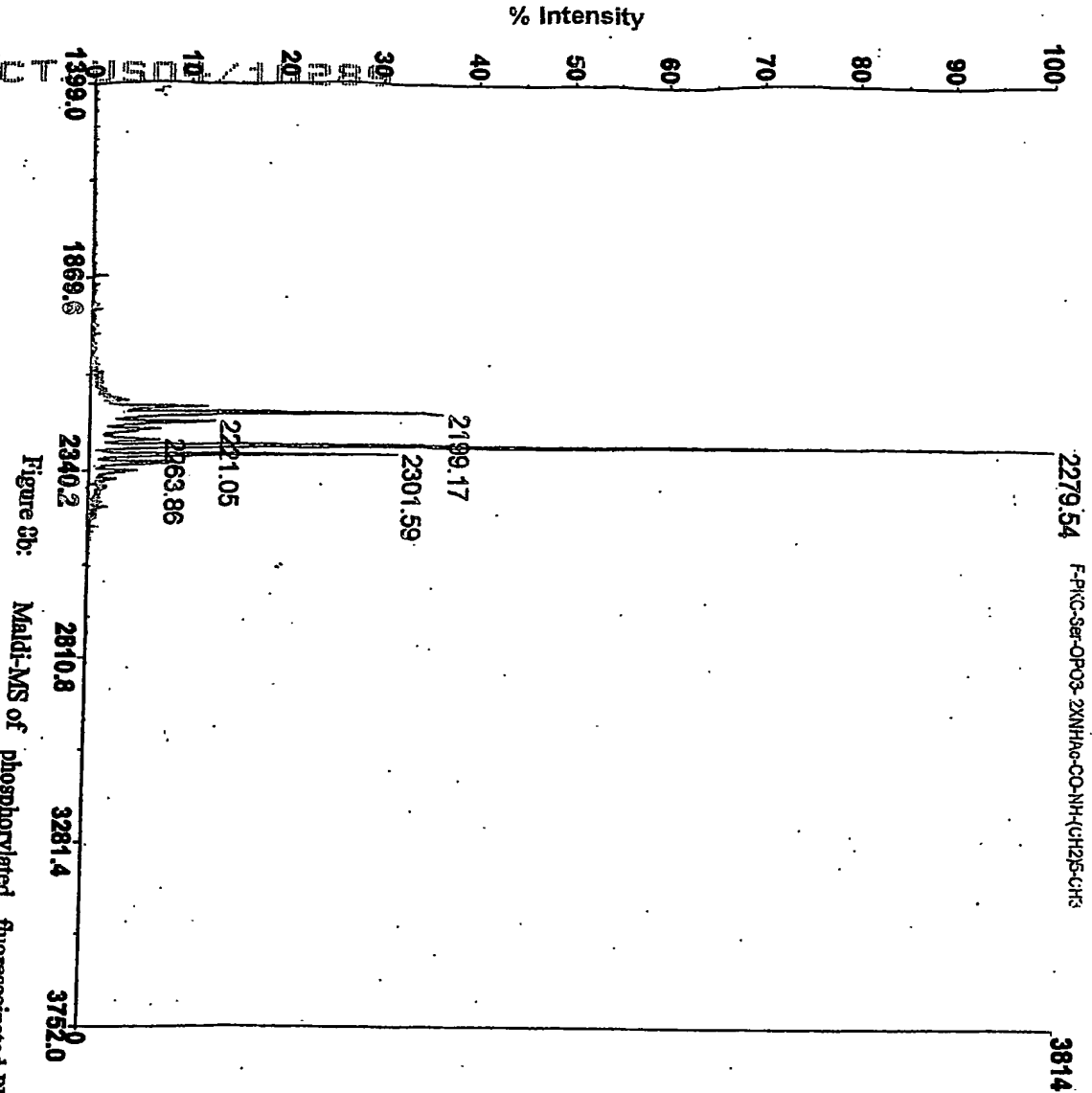


Figure 3b:

Maldi-MS of phosphorylated, fluoresceinated-PKC peptide target that had potential sites blocked as described in Example 1 *before* the addition of multiplexed Nucleation Centers that had been performed from avidin and the heterobifunctional biotin linkers of chemistry I.

Mode of operation: Linear
Extraction mode: Delayed
Polarity: Positive
Acquisition control: Manual

Accelerating voltage: 20000 V
Grid voltage: 95%
Guide wire O: 0.05%
Extraction delay time: 200 nsec

Acquisition mass range: 500 – 5000 Da
Number of laser shots: 100/spectrum
Laser intensity: 1640
Laser Rep Rate: 20.0 Hz
Calibration type: Default
Calibration matrix: a-Cyano-4-hydroxycinnamic acid
Low mass gate: 500 Da

Digitizer start time: 14.258
Bin size: 2 nsec
Number of data points: 16284
Vertical scale: 1000 mV
Vertical offset: 0%
Input bandwidth: 150 MHz

Sample well: 55
Plate ID: JEFF
Serial number: 1197
Instrument name: Voyager-DE
Plate type filename: C:\VOYAGER\100 well plate.plt
Lab name: PE Biosystems

Absolute x-position: 21176.3
Absolute y-position: 20890.3
Relative x-position: -728.201
Relative y-position: -1017.22
Shots in spectrum: 100
Source pressure: 6.258e-007
Mirror pressure: 0
TC2 pressure: 0.00844
TIS gate width: 30
TIS flight length: 940

Applied Biosystems Voyager System 1197

Voyager Spec #1 [BP = 1762.0, 13304]

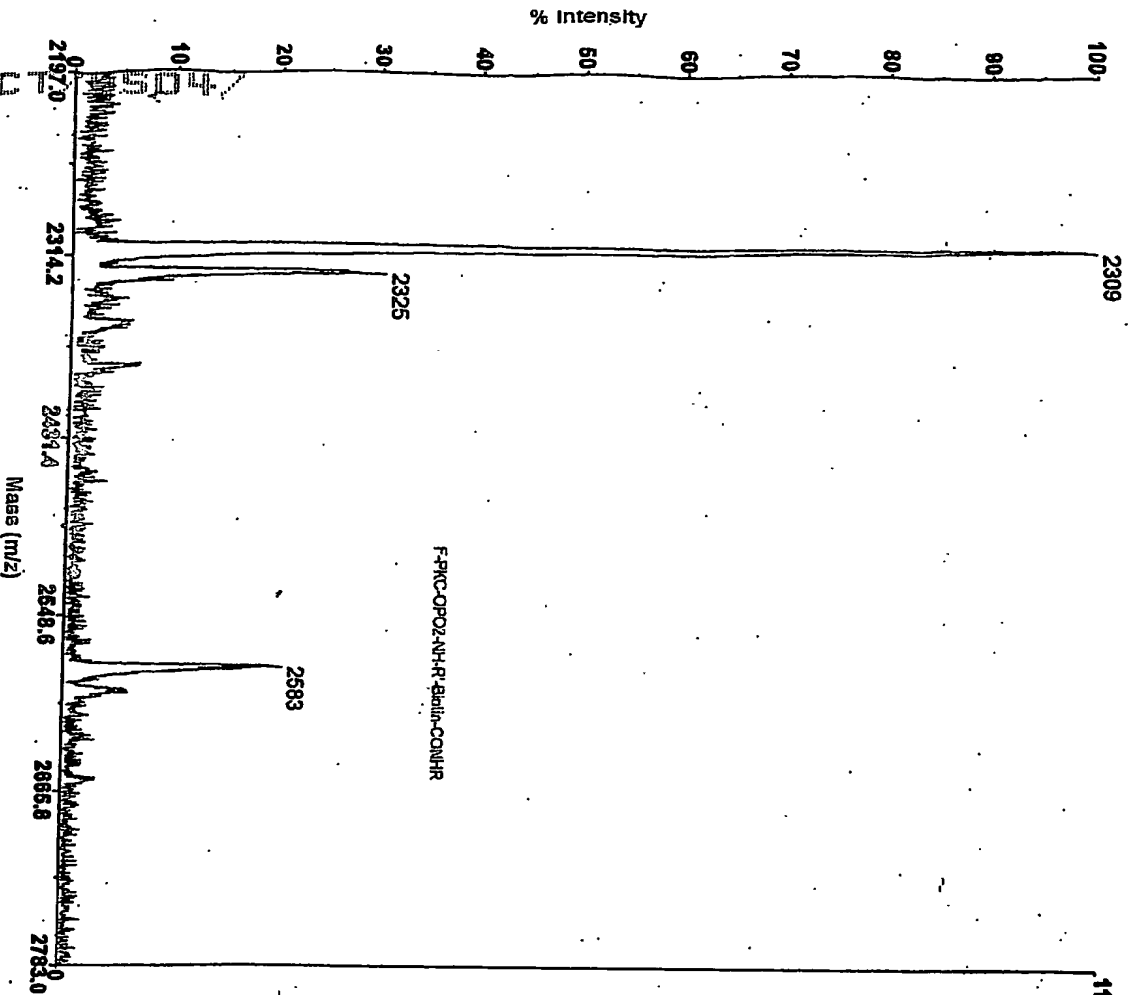


Figure 8c:

Acquired: 1733000, November 19, 2001
 voyager1RAMSBI01nov19_01_0001.dat

Multi-MS of phosphoramidated, fluoresceinated PKC peptide target that had potential reactive sites blocked before the addition of multiplexed preformed Nucleation Centers from avidin and heterobifunctional biotin

Mode of operation: Linear
 Extraction mode: Delayed
 Polarity: Positive
 Acquisition control: Manual

1162.0 Accelerating voltage: 20000 V
 Grid voltage: 95%
 Guide wire On: 0.05%
 Extraction delay time: 200 nsec

Acquisition mass range: 1500 - 3000 Da
 Number of laser shots: 100/spectrum
 Laser intensity: 1981
 Laser Rep Rate: 20.0 Hz
 Calibration type: External - DiVoyager\data/cal2 nov13_0001.cal
 Calibration matrix: p-Cyano-4-hydroxycinnamic acid
 Low mass gate: 1500 Da

Digitizer start time: 24.69
 Bin size: 2 nsec
 Number of data points: 5089
 Vertical scale: 1000 mV
 Vertical offset: 0%
 Input bandwidth: 150 MHz

Sample well: 01
 Plate ID: 1
 Serial number: 1197
 Instrument name: Voyager-DE
 Plate type filename: C:\VOYAGER\100 well plate.plt
 Lab name: PE Biosystems

Absolute X-position: 1883.1
 Absolute Y-position: 47312.8
 Relative X-position: 285.604
 Relative Y-position: 6.30846
 Shots in spectrum: 100
 Source pressure: 4.304e-007
 Mirror pressure: 0
 TC2 pressure: 0.00875
 TIS gate width: 30
 TIS flight length: 840

Printed: 18:03, March 10, 2003

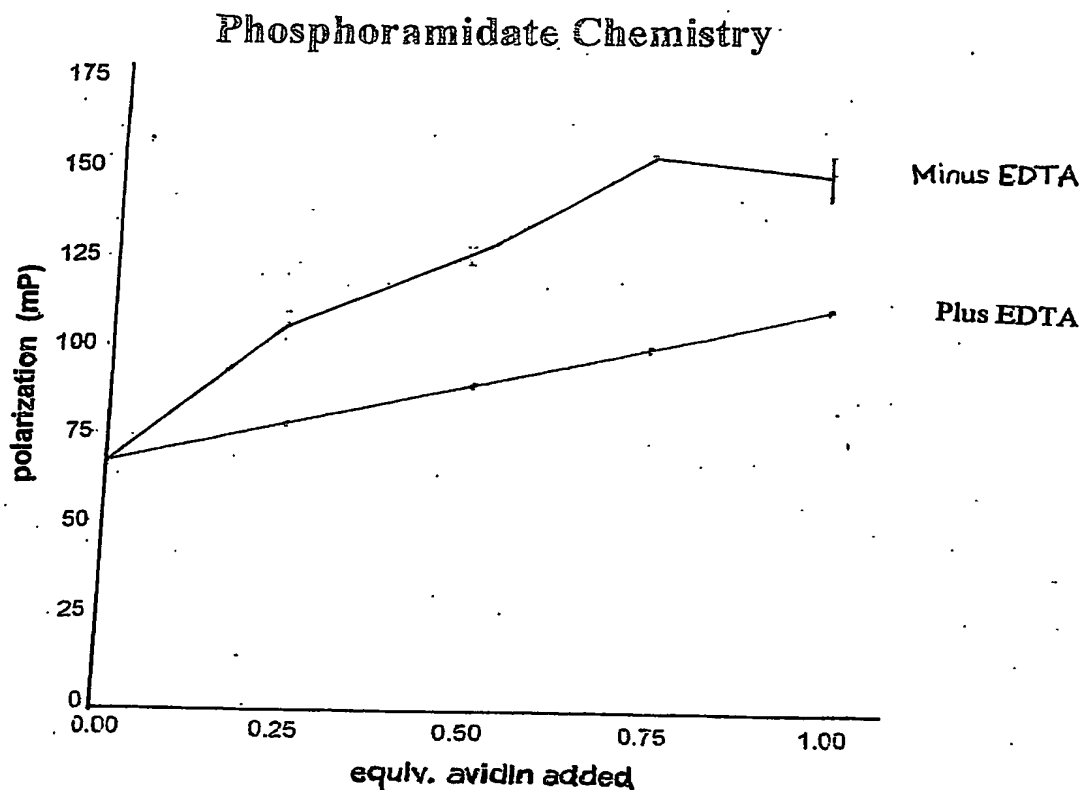


Figure 9. Fluorescence Polarization analysis of the stoichiometry of Nucleation Center Binding. The phosphoramidated PKC peptide target shown in Figure 7 after the addition of varying amounts of multiplexed Nucleation Centers using the linkers of Chemistry I. The two samples differed in that the negative controls were performed in the presence of 5mMolar EDTA which destroys the activity of the kinase.

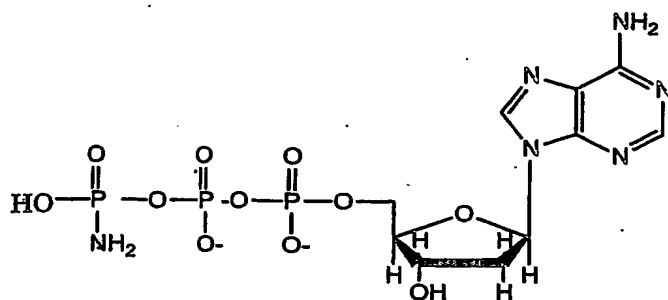
Structure of γ -NH₂-ATP:**7**

Figure 10. Chemical structure of the ATP structural analog, γ -Amino ATP (γ -NH₂-ATP)

Synthesis of γ -Amino-ATP

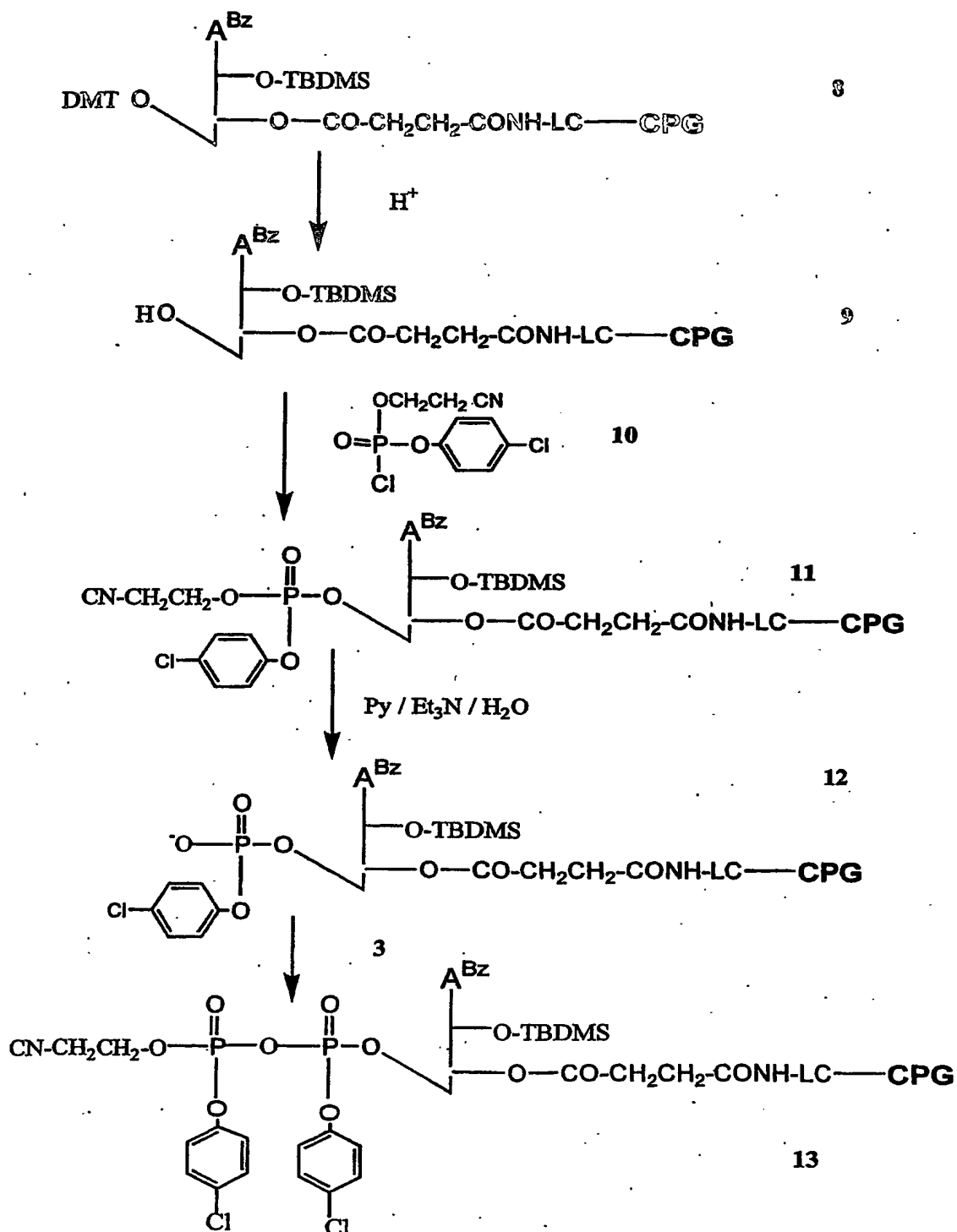


Figure 11. Protocol and chemistry of the present invention for the synthesis of γ -NH₂-ATP

Scheme for the synthesis of gamma-Amino-ATP continues

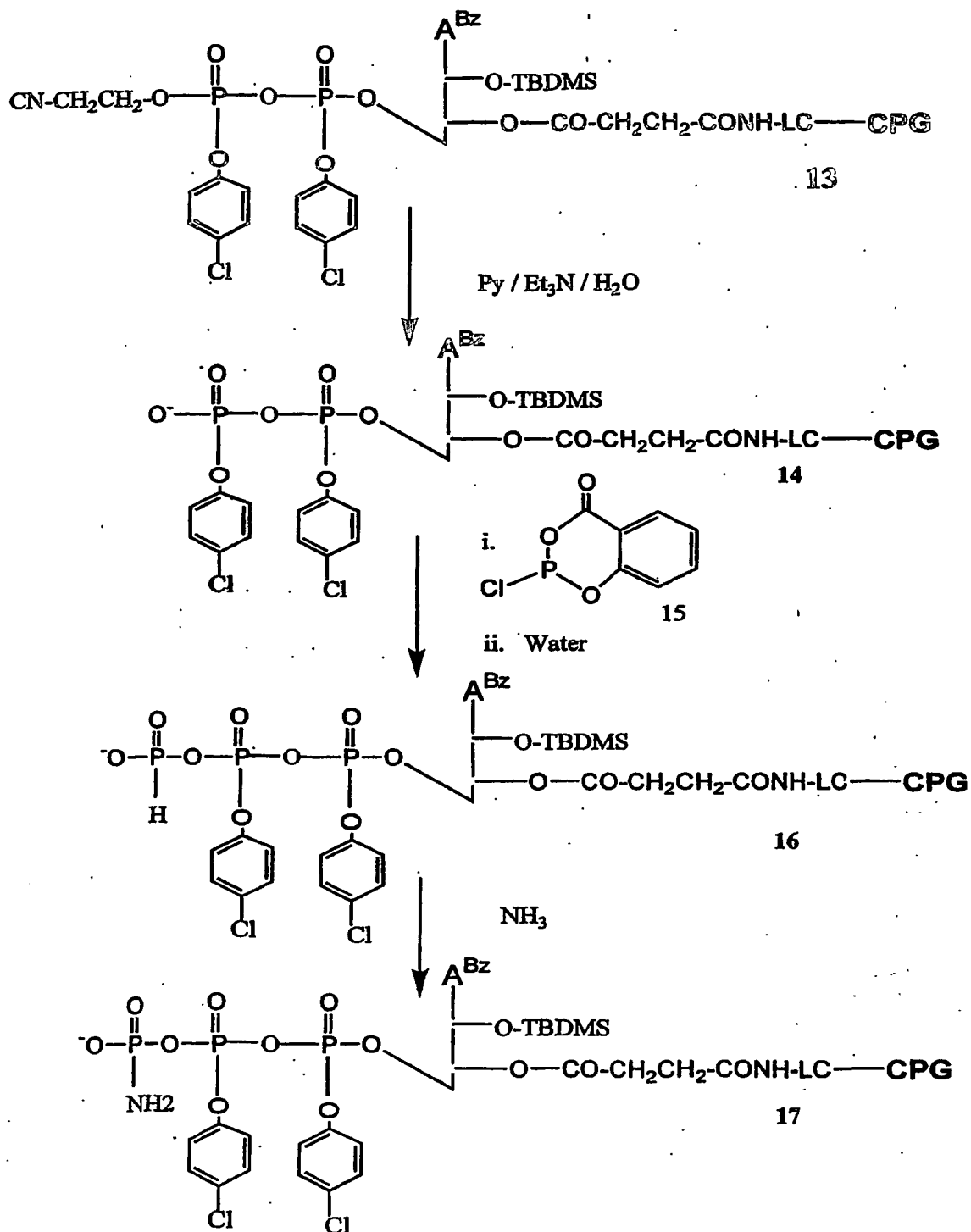
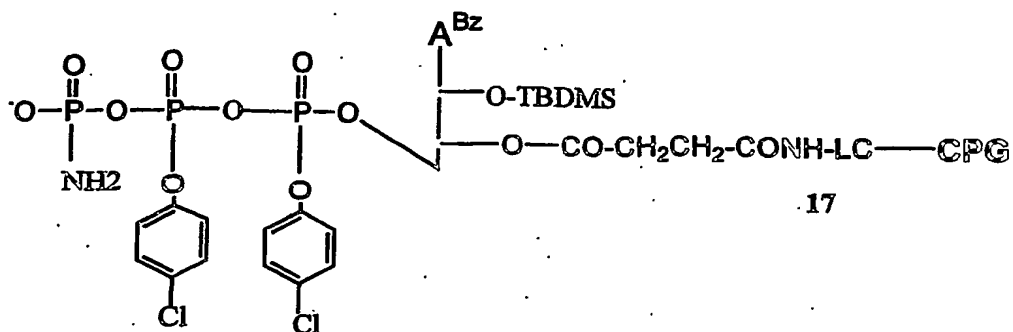
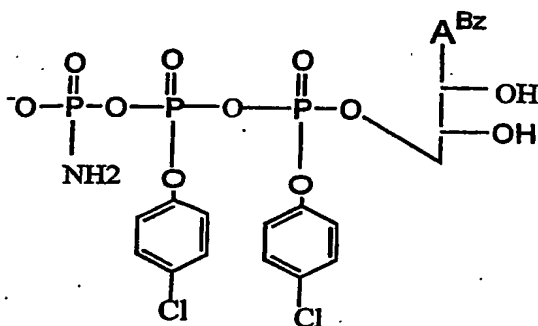


Figure 11: continuation (page 2)

Page 3 of Synthesis of γ -Amino-NH₂

- i. TMG, RT, 4 Hours
ii. NH₄OH, 60°C, 8 hours
iii. Concentrate to dryness under vacuum
iv. TBAF, RT, 16 hours



γ -Amino-NH₂

Figure 11: continuation (page 3)

Alternative Approach For Monitoring the Activity of Protein Kinases

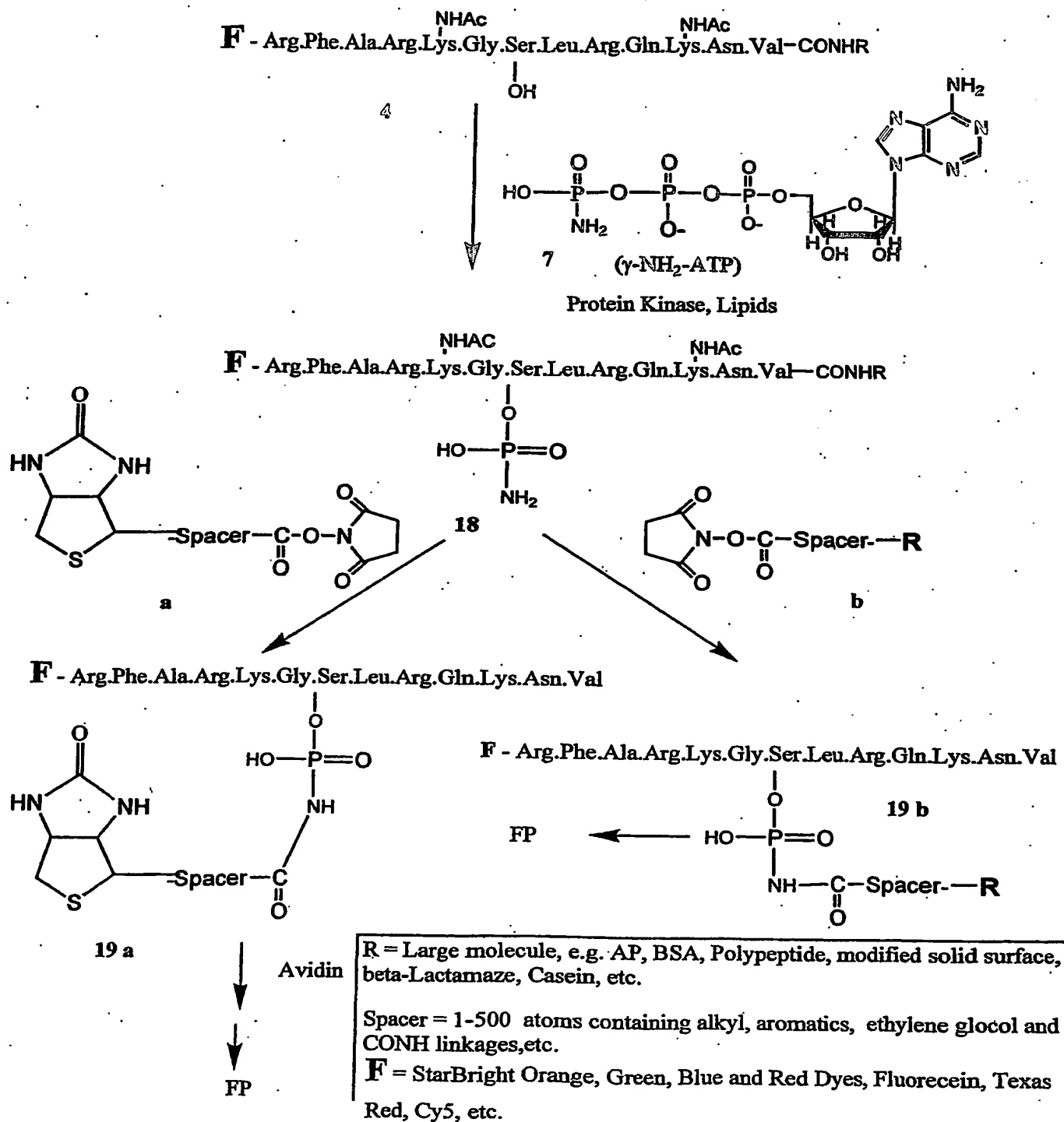
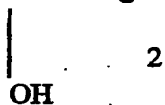


Figure 12. Procedure for phosphoroamidation of fluoresceinated -PKC peptide target using PKC-alpha and $\gamma\text{-NH}_2\text{-ATP}$

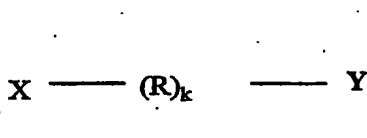
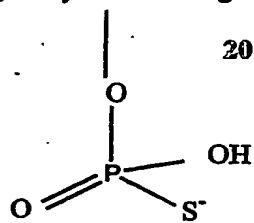
Phosphorothioate Chemistry

F- Arg.Phe.Ala.Arg.Lys.Gly.Ser.Leu.Arg.Gln.Lys.Asn.Val-OH

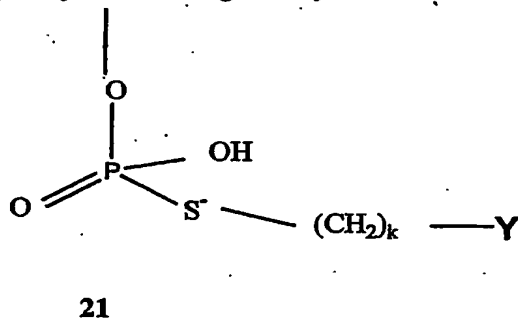


gamma-S-ATP, PKC-alfa

F- Arg.Phe.Ala.Arg.Lys.Gly.Ser.Leu.Arg.Gln.Lys.Asn.Val-OH



F- Arg.Phe.Ala.Arg.Lys.Gly.Ser.Leu.Arg.Gln.Lys.Asn.Val-OH



where k= 1-100

R= Alkyl, alkoxyl, cycloalkanyl,
aromatic, heterocyclic,
ethylene glycolic, peptidyl, etc

Y= Biotin, Biotin-Avidin,
Biotin-Streptavidin, or Large
Polymer such as Alkaline
Phosphatase (AP), Streptavidin
(SA), Casein, glycoprotein, IgG,
enzyme, DNA, RNA with or
without conjugation to Avidin

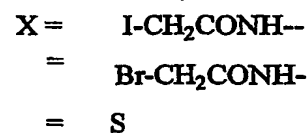
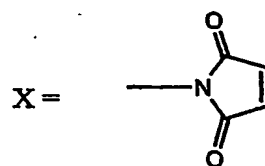
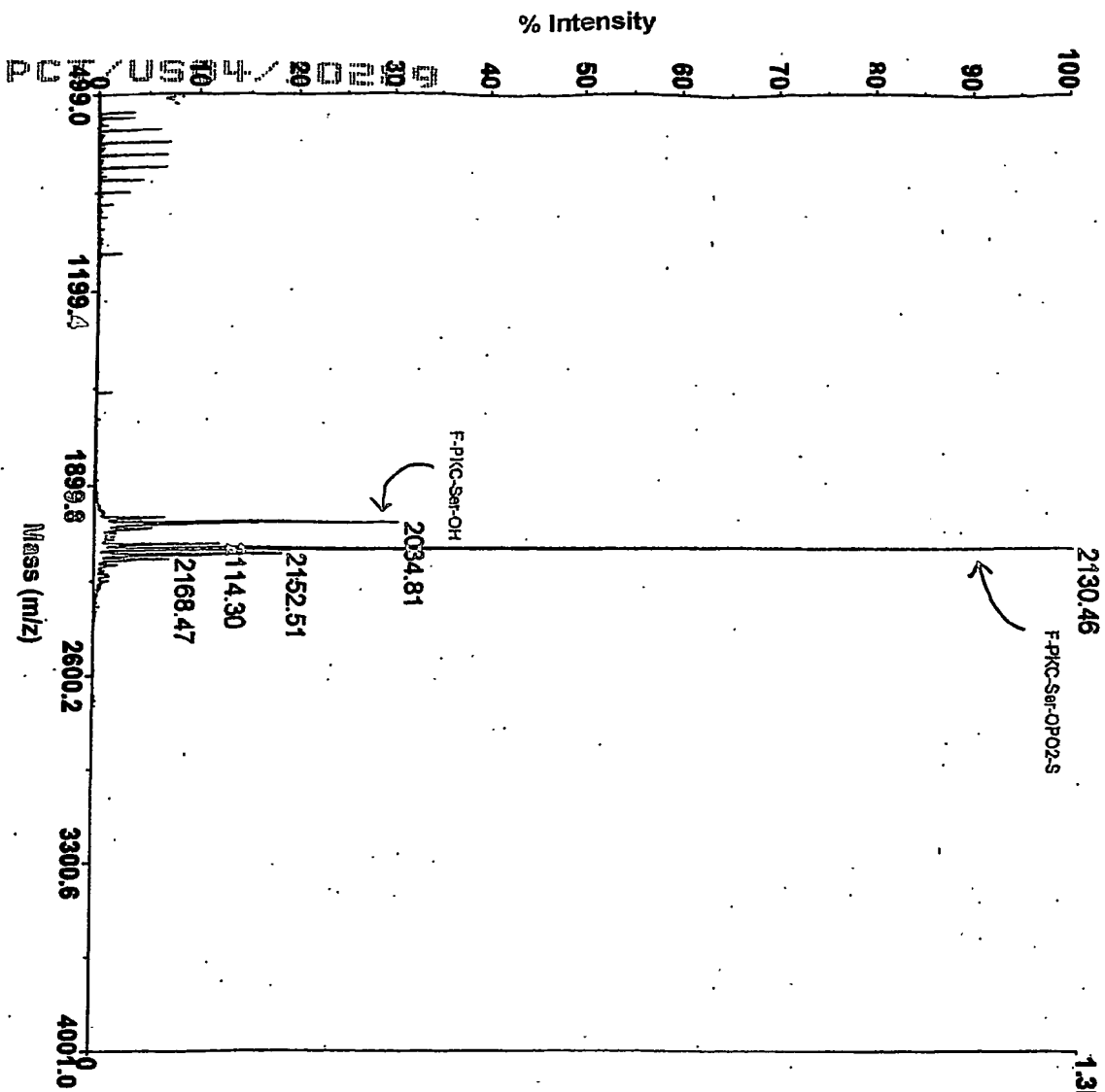


Figure 13. Protocol and chemistry of the present invention for phosphorothiolation and detection of fluoresceinated PKC-peptide target using the single step, nucleation effect rapid assay method and Chemistry III of the present invention

Applied Biosystems Voyager System 1197

Voyager Spec #1[BP = 2130.5, 13023]



Mode of operation: Linear
Extraction mode: Delayed
Polarity: Positive
Acquisition control: Manual

Accelerating voltage: 20000 V
Grid voltage: 95%
Guide wire Q: 0.05%
Extraction delay time: 200 nsec

Acquisition mass range: 600 -- 4000 Da
Number of laser shots: 1000/spectrum
Laser intensity: 1284
Laser Rep Rate: 20.0 Hz
Calibration type: Default
Calibration matrix: s-Cyano-L-hydroxycinnamic acid
Low mass gate: 500 Da

Digitizer start time: 14.298
Bin size: 2 nsec
Number of data points: 12960
Vertical scale: 1000 mV
Vertical offset: 0%
Input bandwidth: 150 MHz

Sample well: 23
Plate ID: PLATE1
Serial number: 1197
Instrument name: Voyager-DE
Plate type filename: C:\VOYAGER\100 well plate.plt
Lab name: PE Biosystems

Abscissa x-position: 11746.9
Relative x-position: 37147.9
Relative y-position: -0.690595
Shots in spectrum: 0.398375
Source pressure: 100
Mirror pressure: 7.869e-007
TIS gate width: 0
TIS flight length: 0.01229
940

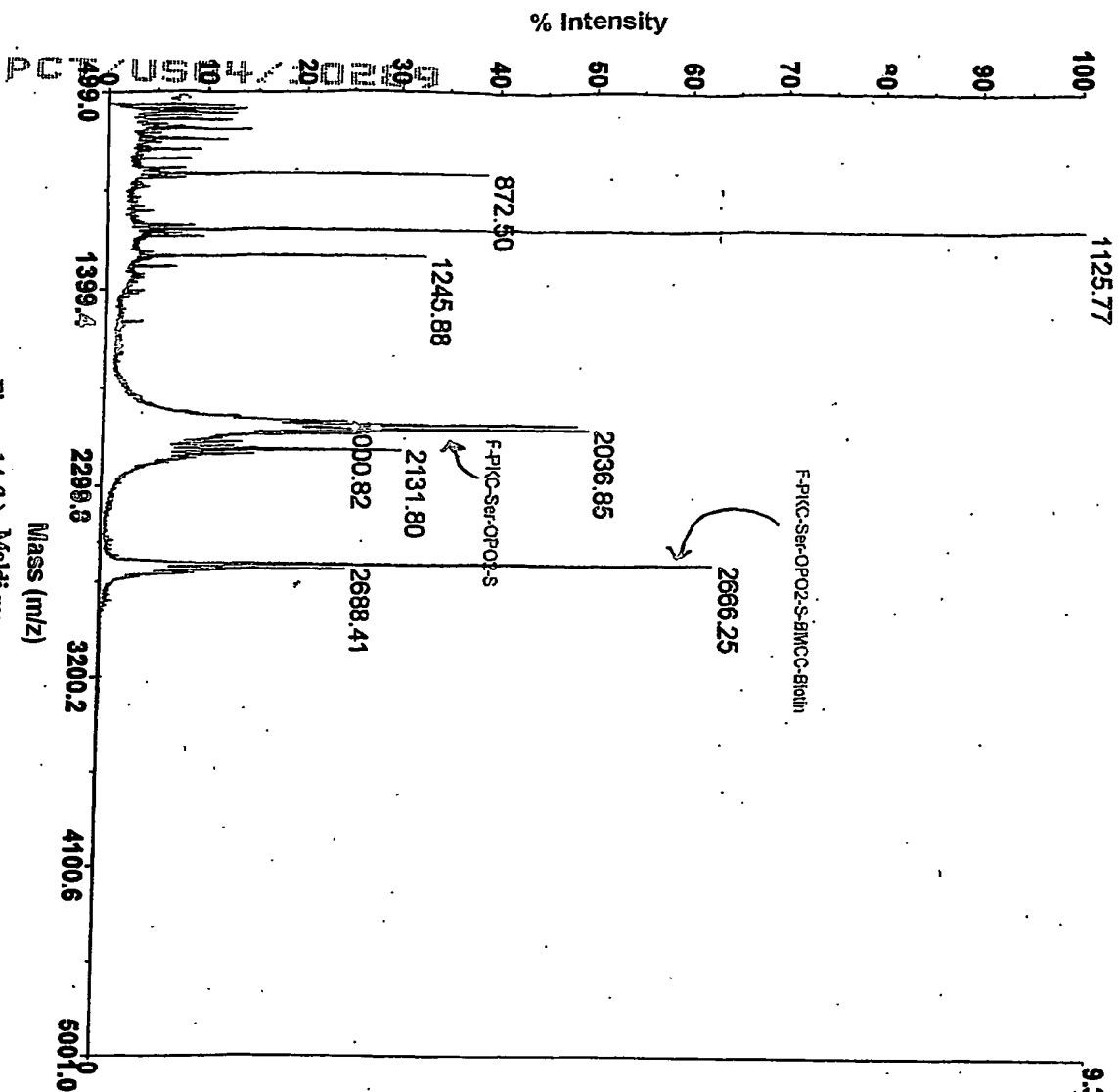
Acquired: 13:57:00, September 21, 2001
File: \vgs\acdata\PE886-11Bonet\32_0001.dat

Figure 14 (a): MALDI-MS of Phosphorothiolated fluoresceinated -PKC peptide target

Printed: 16:24, March 11, 2003

Applied Biosystems Voyager System 1197

Voyager Spec #11BP = 1125.6, 9311]



Acquired: 17/10/00, September 21, 2001

Acquired data: RB5558-11-B1_0002.dct

Figure 14 (b):

Maldi mass spectrum of the same phosphorothiolated, fluoresceinated PKC peptide target 15 minutes after addition of equimolar equivalents multiplexed Nucleation Centers preformed from avidin and the hetero-bifunctional biotin.

Mode of operation: Linear
Extraction mode: Delayed
Polarity: Positive
Acquisition control: Manual

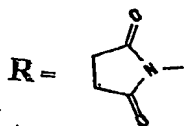
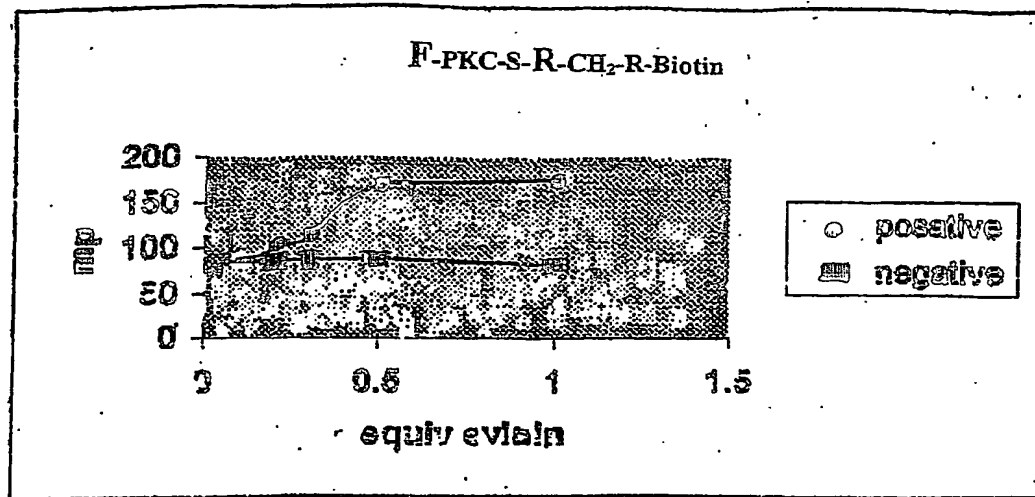
Accelerating voltage: 20000 V
Grid voltage: 95%
Guide wire on: 0.05%
Extraction delay time: 200 nsec

Acquisition mass range: 500 - 5000 Da
Number of laser shots: 100/spectrum
Laser intensity: 1640
Laser Rep Rate: 20.0 Hz
Calibration type: Default
Calibration matrix: e-Cyano-4-hydroxycinnamic acid
Low mass gate: 500 Da

Digitizer start time: 14.288
Bin size: 2 nsec
Number of data points: 15324
Vertical scale: 1000 mV
Vertical offset: 0%
Input bandwidth: 150 MHz

Sample well: 47
Plate ID: RB109FEB21
Serial number: 1197
Instrument name: Voyager-DE
Plate type filename: C:\VOYAGER\100 well plate.pil
Lab name: PE Biosystems

Absolute x-position: 32051.3
Absolute y-position: 26104.1
Relative x-position: -16.1985
Relative y-position: -883.367
Shots in spectrum: 100
Source pressure: 9.673e-007
Mirror pressure: 0
TC2 pressure: 0.01058
TIS gate width: 30
TIS flight length: 940



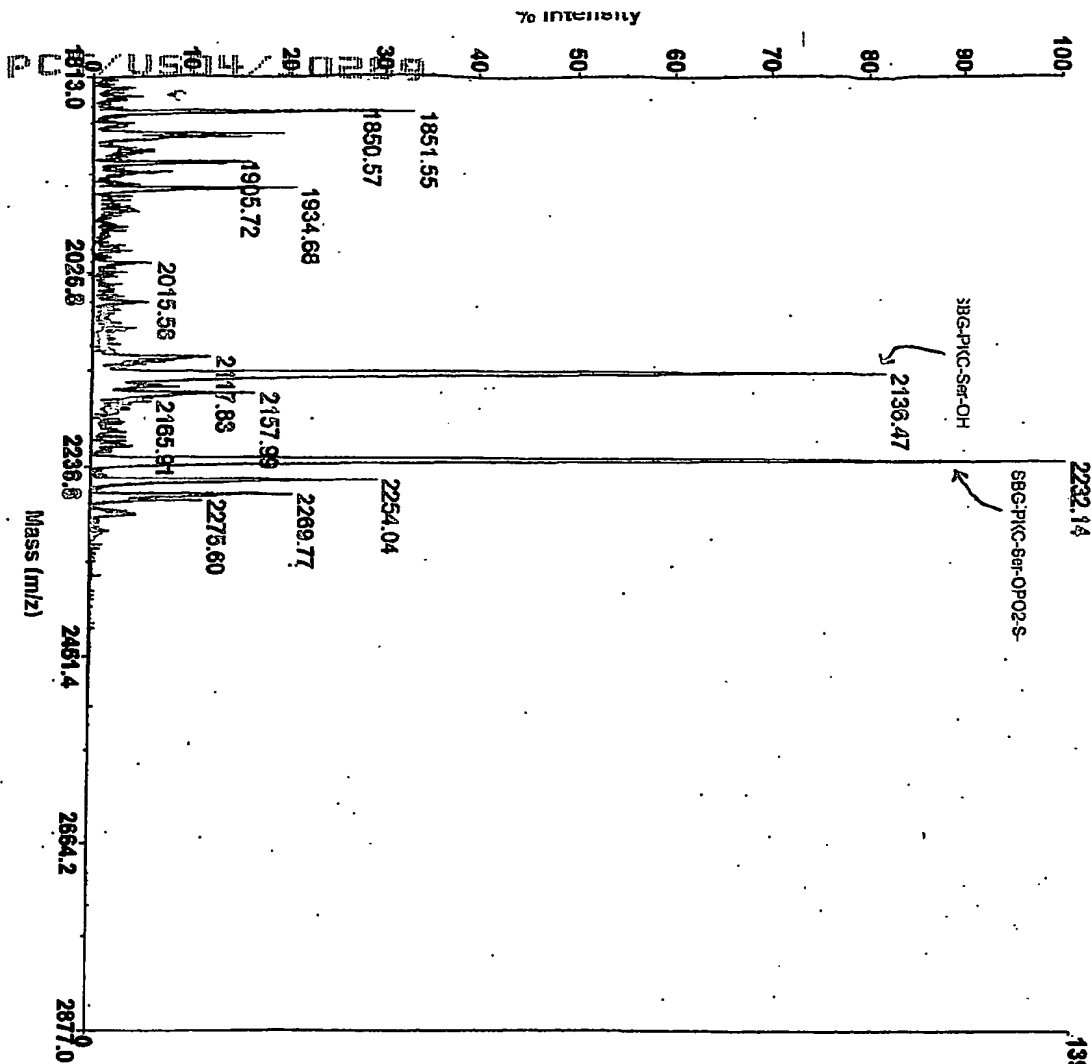
$R = \text{Long Chain alkyl}$

Figure 14 (C) Fluorescence polarization analysis of the same sample used to generate the spectrum of 14(b), above, showing the titration with multiplexed Nucleation Centers that were prepared from avidin and the hetero-bifunctional linkers, maleimido BMCC-biotin.

ADOC 37B71VAV TSEB

Applied Biosystems Voyager System 1107

Voyager Spec #11BP = 2231.9, 13981



Mode of operation: Linear
Extraction mode: Delayed
Polarity: Positive
Acquisition control: Manual

1398.0 Accelerating voltage: 20000 V
Grid voltage: 95%
Guide wire 0: 0.05%
Extraction delay time: 200 nsec

Acquisition mass range: 1000 - 4000 Da
Number of laser shots: 1000/spectrum
Laser intensity: 1319
Laser Rep. Rate: 20.0 Hz
Calibration type: Default
Calibration matrix: e-Cyano-4-hydroxycinnamic acid
Low mass gate: 1000 Da

Diluter alert time: 20.17
BIN size: 2 nsec
Number of data points: 10023
Vertical scale: 1000 mV
Vertical offset: 0%
Input bandwidth: 150 MHz

Sample well: 24
Plate ID: RB109FEB23
Serial number: 1197
Instrument name: Voyager-DE
Plate type filename: C:\VOYAGER\100 well plate.plt
Lab name: PE Biosystems

Absolute X-position: 16216.2
Absolute Y-position: 37102.9
Relative X-position: -611.266
Relative Y-position: -44.6175
Shots in spectrum: 100
Source pressure: 1.34e-006
Mirror pressure: 0
TIC2 pressure: 0.01205
TIS gate width: 30
TIS flight length: 940

Figure 15 (b): MS of Phosphorylated StarBright Green -PKC peptide target with enzyme and γ-S-ATP

Applied Biosystems Voyager System 1107

F-PKC-Voyager Spec #11BP = 1694.6, 104831

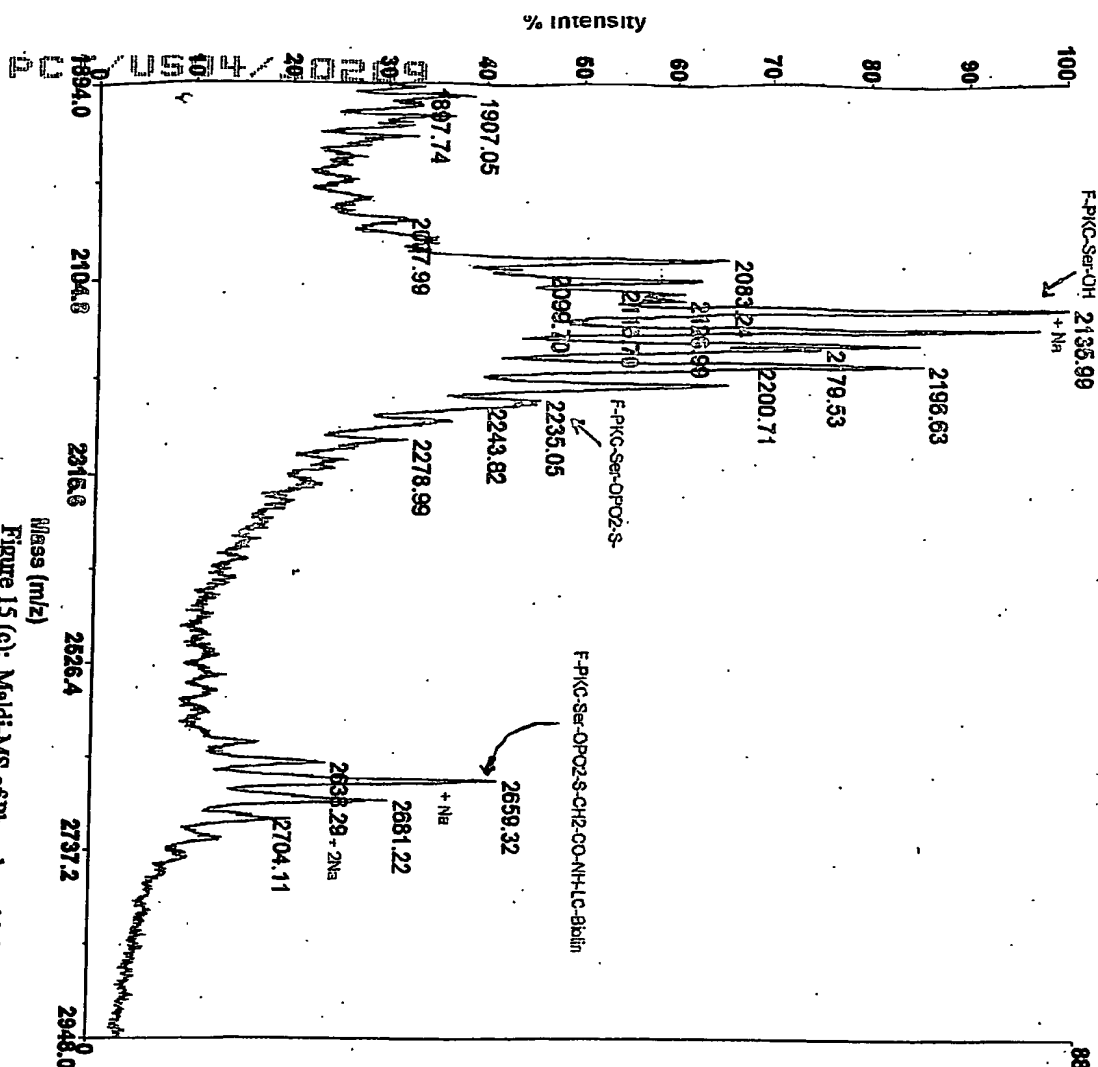


Figure 1.5 (c):

Maldi-MS of Phosphorylated StarBright Green -PKC peptide target
After the addition of multiplexed Nucleation Centers performed from
Biotin and the maleimido-heterobifunctional linker

Mode of operation:	Linear
Extraction mode:	Delayed
Polarity:	Positive
Acquisition control:	Manual
Accelerating voltage:	20000 V
Grid voltage:	95%
Guide wire O:	0.05%
Extraction delay time:	200 nsec
Acquisition mass range:	1000 - 3500 Da
Number of laser shots:	100/spectrum
Laser intensity:	1747
Laser Rep Rate:	20.0 Hz
Calibration type:	Default
Calibration matrix:	a-Cyano-4-hydroxycinnamic acid
Low mass gate:	1000 Da
Digitizer start time:	20.17
Bin size:	2 nsec
Number of data points:	8729
Vertical scale:	1000 mV
Vertical offset:	0%
Input bandwidth:	150 MHz
Sample well:	24
Plate ID:	RB109FEB23
Serial number:	1197
Instrument name:	Voyager-DE
Plate type filename:	C:\VOYAGER\1100 well plate.plt
Lab name:	PE Biosystems
Absolute x-position:	17820.6
Absolute y-position:	37815
Relative x-position:	793.118
Relative y-position:	687.548
Shots in spectrum:	100
Source pressure:	1.316e-008
Mirror pressure:	0
TC2 pressure:	0.0114
TIS gate width:	30
TIS flight length:	940

Fluorescence Polarization

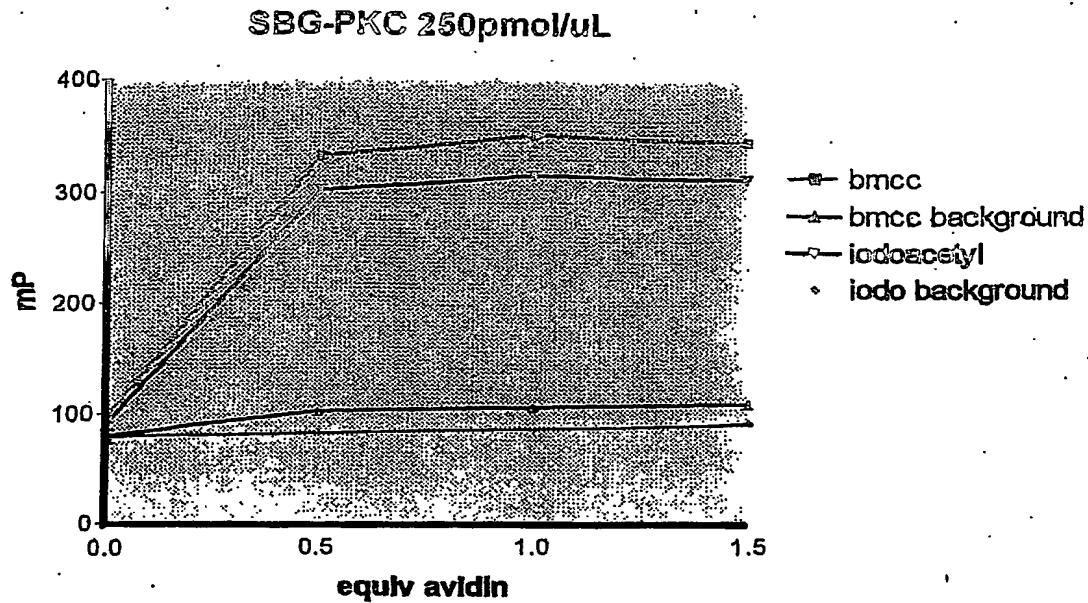
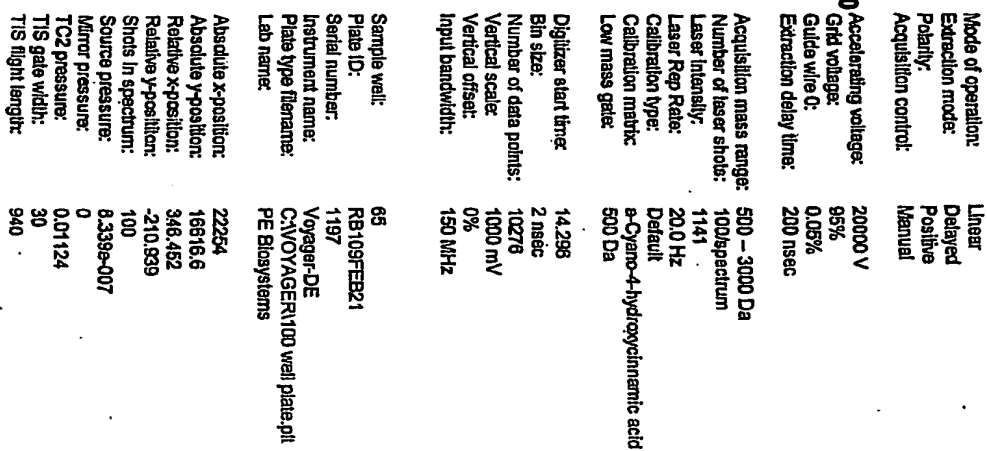


Figure 16. Fluorescence Polarization Analysis of the extent of the reaction of multiplexed Nucleation Centers prefomed from avidin and multiple heterobifunctional linkers bearing biotin at one terminus and maleimido- (blue line) and iodoacetamido- reactive groups at the other (purple line). The StarBright Green -PKC peptide target was phosphorylated by PKC-theta using γ -S-ATP as the donor.

Voyager Spec #1 [BP = 2187.1, 2839]



Arch 12, 2003

Voyager Spec #1[BP = 2277.8, 1326]

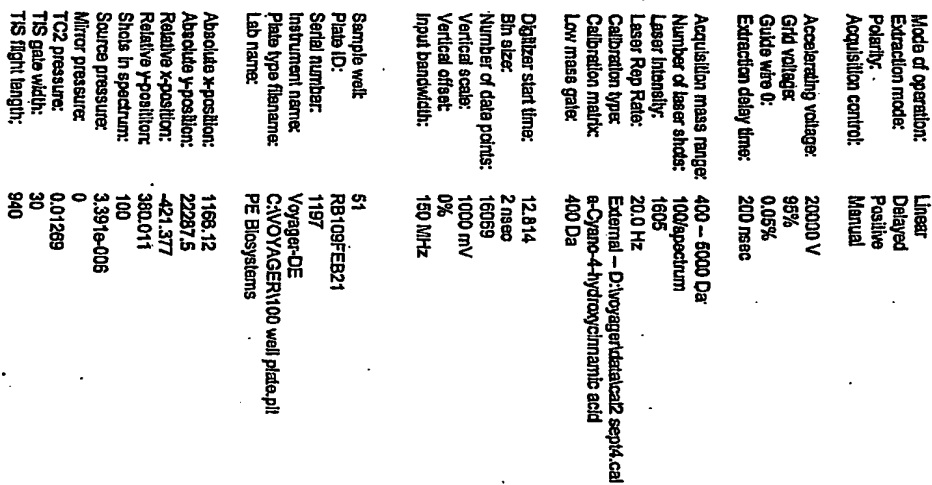
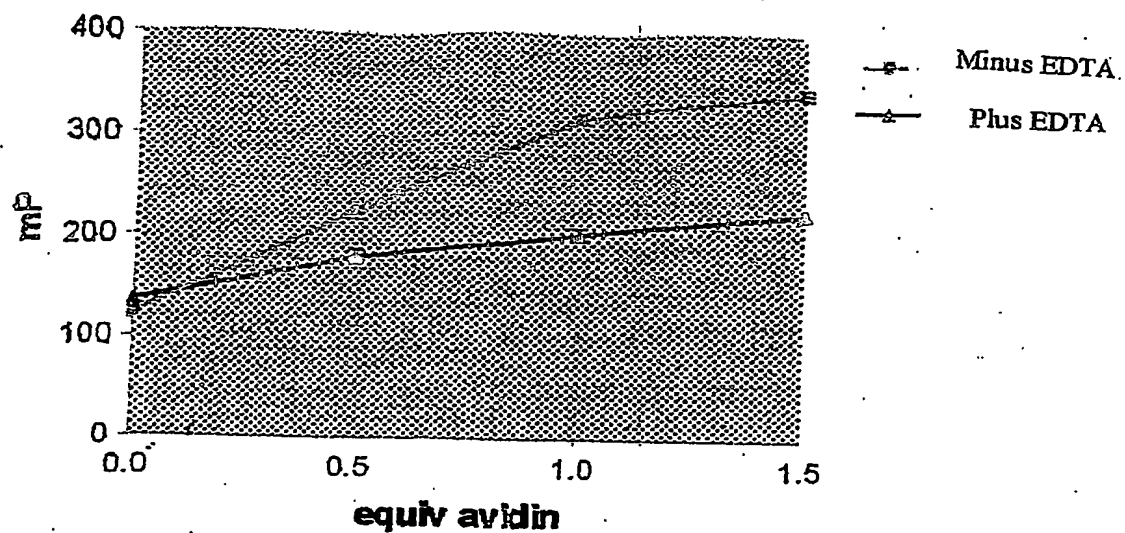


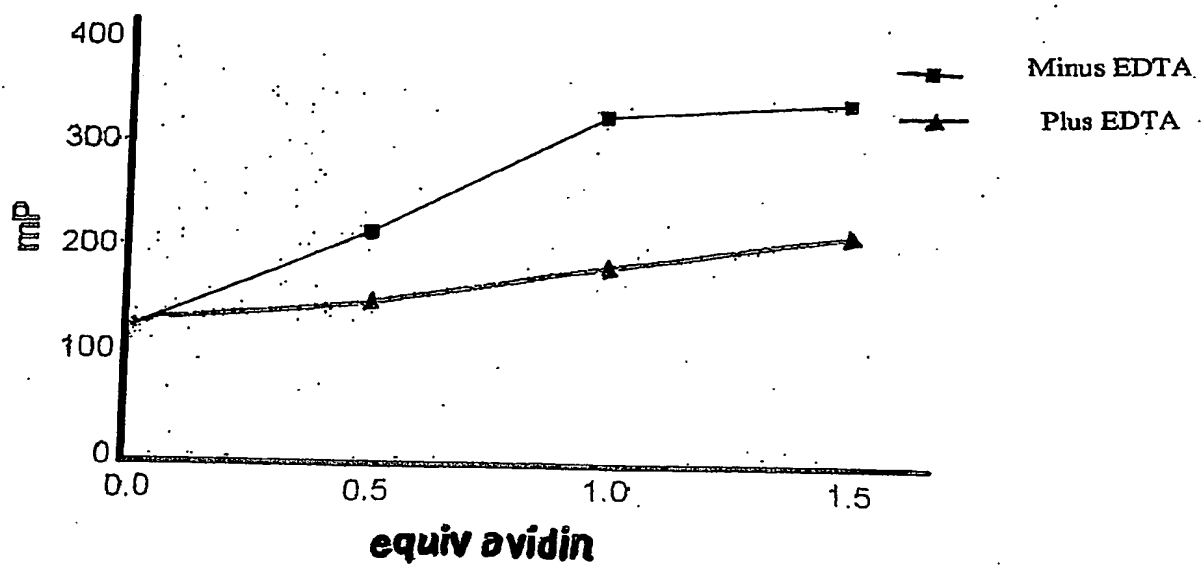
Figure 17 (b): MALDI-MS of Phosphothiolated StarBright Green -PKC peptide target

Figure 18 (a) : Fluorescence Polarization of SBO-PKC-Ser-OPO2-S-BMCC-LC-Biotin after the addition of Avidin



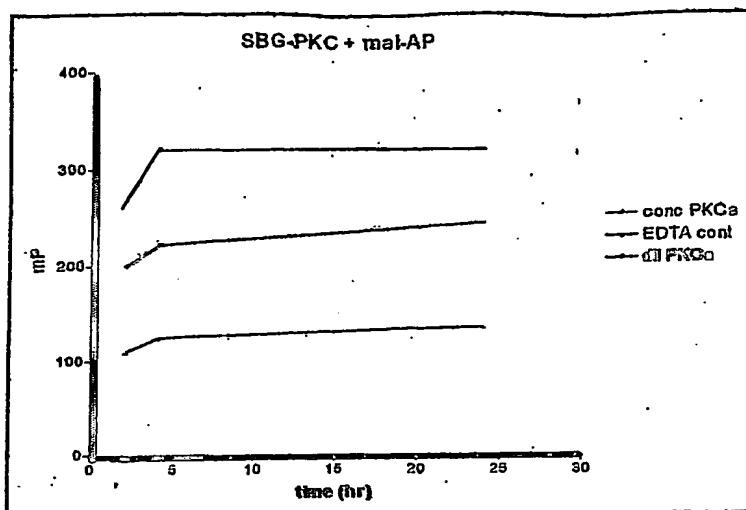
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Figure 18 (b) : Fluorescence Polarization of SBO-PKC-Ser-OPO2-S-Iodoacetyl-LC-Biotin after the addition of Avidine



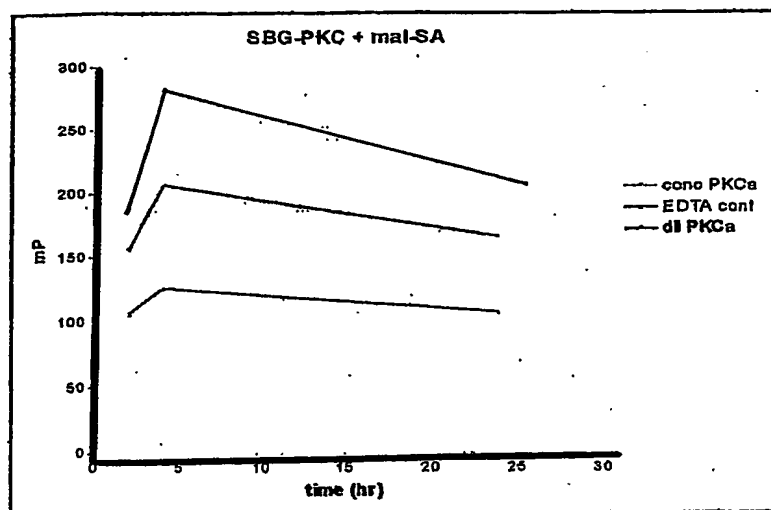
Fluorescence Polarization Using Large Molecules

(a)



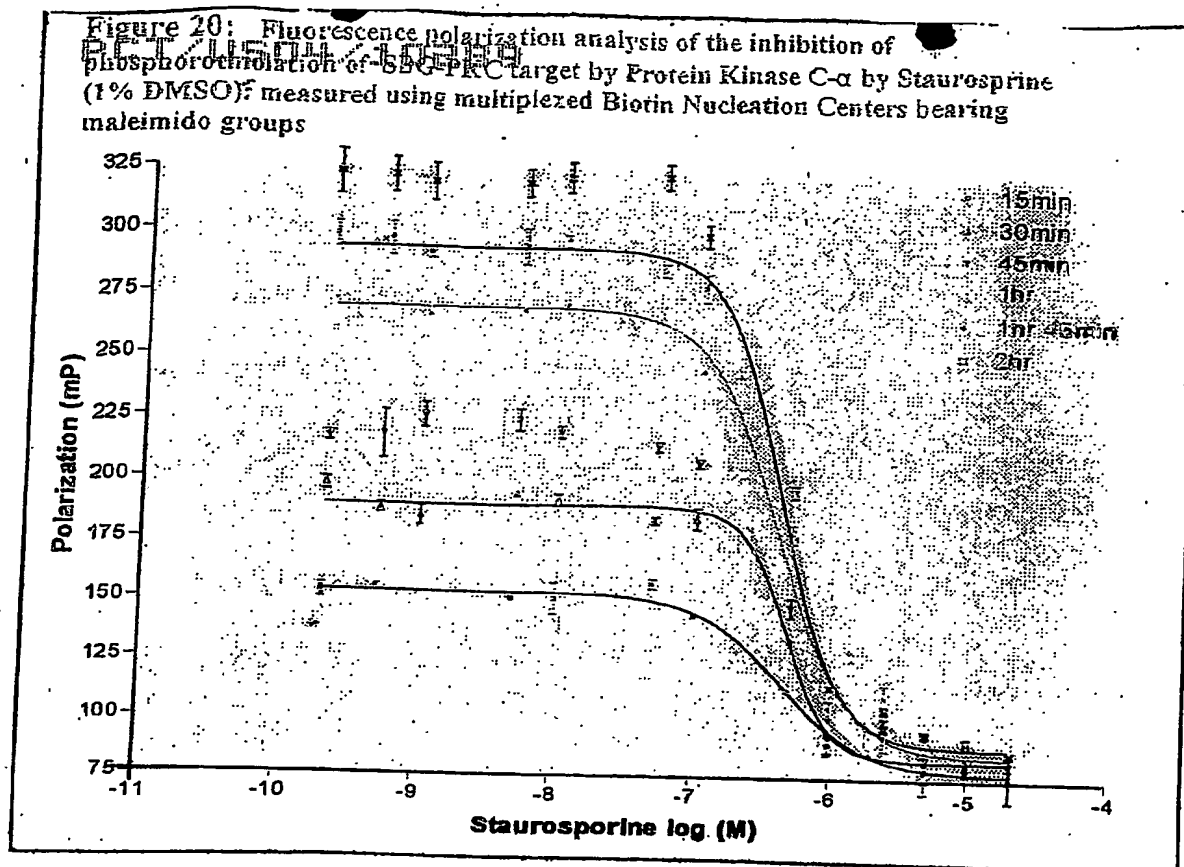
time (hr)	conc PKCa	EDTA cont	dil PKCa
2	267	110	200
4	321	125	223
24	321	136	245

(b)



time (hr)	conc PKCa	EDTA cont	dil PKCa
2	187	106	156
4	281	126	207
24	213	107	166

Figure 19: Fluorescence polarization of analysis of phosphorothiolated SBG-PKC after the addition of multiplexed Nucleation centers comprised of Alkaline Phosphatase, figure (a), and Streptavidine, figure (b), bearing multiple maleimido groups capable of reacting with the phosphorothiolated peptide described in figure 13.



EC50	4.39E-07	4.76E-07	4.40E-07	4.01E-07	4.16E-07	4.39E-07
KI	1.26E-07	1.36E-07	1.26E-07	1.15E-07	1.19E-07	1.26E-07

Fluorescence Polarization (mp)

15min	30min	45min	1hr	1hr45	2hr
67	76	65	71	80	87
80	78	80	92	91	93
87	69	83	85	93	93
87	99	95	107	105	103
93	85	93	99	108	103
113	111	143	167	174	114
142	143	208	243	283	193
154	158	211	267	282	303
143	156	216	277	288	327
150	149	228	270	300	327
151	152	230	282	291	313
155	152	207	268	289	326
155	149	218	276	289	314
149	154	217	271	302	331
74	78	74	82	75	82
152	145	212	263	282	307

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